REMARKS

Reconsideration of this application is respectfully requested.

Separate sheets showing amendments made to the claims set forth above are attached hereto in the attachment entitled "VERSION WITH MARKINGS TO SHOW CHANGES MADE."

Specification

Requirement under 37 CFR 1.125(a)

As required pursuant to 37 CFR 1.125(a), the applicant submits herewith a substitute specification, which incorporates the amendments effected in the preliminary amendment recorded on February 13, 2001. The substitute specification does not include any new subject matter.

Claim Rejections - 35 U.S.C. §102

Rejection under 35 U.S.C. §102(b)

Claims 1-10 have been rejected under 35 U.S.C. §102(b) as being anticipated by Fahy (WO 96/30545).

In view of the amendments to claim 1 as outlined above, Applicant respectfully submits that the method as claimed clearly distinguishes over the teachings of Fahy. Accordingly, reconsideration of the objections raised in the Office Action is respectfully requested.

In reply to the objections raised in the Office Action, the applicant submits that the teachings of Fahy are only of general interest, and are significantly deficient and fail to provide an anticipation of the present invention in amended claim 1. The applicant respectfully submits that Fahy's method is distinctly and significantly different, in both principle and technical design, to the teachings of the present invention.

In connection with the rejection of claims 1, 2 and 8, the applicant submits that Fahy's method and the primer specific mispair extension assay (PSMEA) method described in the present application differ fundamentally, and are based on different technical principles and concepts.

More specifically, Fahy provides a method for analyzing or essentially distinguishing between wild-type and corresponding mutant sequences by comparison of primer extension length. In contrast, the present invention teaches the determination of specific genotypes based on an analysis of specific and mispair primer extension, and allows for the discrimination between genotypes which may vary by only a single nucleotide base (i.e. HCV 1a and 1b); and also allows for the determination of nucleotide insertions, as seen in Fig 2 with CA insertion in HCV 6a subtype, which will differentiate between 6a and other genotypes; for the detection of multiple nucleotide variations, as noted on page 13 for HCV 3a and 3b; and for the discrimination between genotypes based on their primer specific pair and mispair extension analysis.

In addition, Fahy's method cannot differentiate between low level variant viral species or low level heterozygote species. The present method also enables a sensitive detection of low levels of heterozygotes as noted on page 14, and for the highly accurate analysis of mixed infections by different genotypes, and may additionally be used for large cohort studies. The scope of Fahy's method does not enable such determinations.

The Office Action further asserts that Fahy provides a primer that is complementary to the identical region of a nucleotide sequence. However, the Applicant respectfully submits that this is but another difference between the teachings of Fahy and the teachings of the present invention. More specifically, the primers used in the present method are complementary to the divergent or variation region, (i.e. the type specific region, for example, the sequence from -176 to -157 in 5' untranslated region is different for the various HCV genotypes) and extend into the identical region, or may also overlap the variable and identical regions of a genotype. Primer extension is then stopped at the 3' end of the extended region due to the mismatched pairs existing just at and/or just after the mutation/variation point(s) to be detected. Conversely, Fahy's primers are complementary to the identical region and extend into the divergent region, as noted on page 7, lines 28-32, and primer extension stops by the incorporation of chain termination dideoxynucleotides (ddNTPs) in primer extension.

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Moreover, the Office Action notes that Fahy's method uses dNTPs in the primer extension reaction. However, due to the principles upon which Fahy's method is based, the use of ddNTPs is required. In fact, Fahy notes that his method is based on the polymerase-directed extension of a primer using "*selected mixtures of up to three nucleoside triphosphates and one or more chain terminating, base pairing entities, such as dideoxynucleoside triphosphates*" i.e. ddNTPs (page 6, lines 31-36, and throughout description and claims). In fact, all of Fahy's noted examples all use ddNTPs, and Fahy further notes the preferred use of ddNTP's (page 12, line 35) in his method. In contrast, the present method, as claimed in claim 1, the extension reaction is carried out in the absence of any ddNTPs.

Fahy does suggest on page 8, line 6, that an alternative embodiment containing no ddNTPs can be used, however, Fahy does not provide the conditions for such use, i.e. the polymerase that would allow his method to work in the absence of ddNTPs, nor does Fahy provide any examples of experimental data where no ddNTPs are used. Since Fahy does not provide details to experiments lacking ddNTPs, it is unclear how Fahy's method would accommodate the lack of ddNTPs. In addition, since Fahy's method does not consider mispair extension, and only examines extension products based on their lengths, Fahy's method could not differentiate between such genotypes, even when using an enzyme that may be used in practicing the present method. For example, if Fahy were to exclude the use ddNTPs from his method, one skilled in the art would expect that the length of the extended products generated from different genotypes, with, for example, a single nucleotide difference would be of the same length. Therefore, if Fahy did omit the use of ddNTPs from his method, he could not differentiate between genotypes with a single nucleotide variation. That is to say, the only approach Fahy provides for the detection of a single mutation is to use exo+ULTma DNA polymerase in the presence of ddNTPs. Accordingly, in connection with the Office Action's position relating to Fahy's use of an incomplete set of dNTPs, Fahy's method is distinctly different from the teachings of the present invention, and additionally teaches away from a method that excludes the use of ddNTPs.

With respect to separation based on extension product length, The Office Action is correct in noting that Fahy's method is based on comparison and analysis of primer lengths to differentiate between mutant and wildtype sequences. Fahy states on page 6, lines 31-36, that "*the present invention is based on polymerase-directed extension of an oligonucleotide primer*

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using selected mixtures of up to three nucleoside triphosphates (dNTPs) and one or more chain terminating, base pairing entities, such as dideoxynucleoside triphosphates (ddNTPs)". Therefore, the size of the extended primer, extended by matched pair formation and extension, is only a marker for distinguishing between a wild genotype and mutant genotype. Therefore, as noted in the Office Action, Fahy compares primer lengths to distinguish between genotypes and fails to consider mispair formation in primer extension. In contrast, the method of the present invention examines genotype-specific primer pair and mispair extension to identify the mutant, wild type and other genotypes, based on extension termination by mispair(s) and both nucleotide-specific pairing and mispairing as important markers for specific genotype determination.

Therefore, in view of the present invention, Fahy's analysis of a primer extension product length is clearly deficient, because primer extension length will vary dependent on various factors, such as what nucleotide combinations are used, or on the flanking sequence about a mutation. In addition, it has been demonstrated that many different sizes of extended primers occur, depending on which bases are added. For example, it is well known that the incorporation efficiency of G and C is significantly lower than the incorporation efficiency of A and T, whereby primer extension could be terminated during DNA chain elongation by the presence of several consecutive C or G incorporations (Haff et al. 1997, Genome Research, 7:378-388; Hu, et al. 1998, Nucleic Acids Res. 26:5013-5015). It has also been demonstrated that an extended primer may have different lengths on a template due to mispair extension and the incorporation efficiency of variable dNTPs (Hu et al, 1998). In addition, the length of the predominant (strongest) DNA bands (visible bands in a gel electrophoresis) generated from two genotypes could be identical when using some DNA polymerases.

Therefore, since many factors are involved in varying the size of the extended primer, Fahy's method of simply examining the size of the primer extension products to identify a mutant or wildtype genotypes, or to identify a single base point mutation difference is deficient. In contrast, the method of the present invention overcomes the deficiencies and problems of Fahy's method by analyzing genotypes based on specific-pairing and misparing primer extension using an appropriate enzyme. This is distinctly different and more reliable than the teachings of Fahy.

In connection with the assertion that Fahy uses high fidelity polymerase enzymes with 3'-5' exonuclease activity, the applicant respectfully submits that the DNA polymerases with 3'-5' proofreading activity suggested in Fahy's method may be proofreading polymerases, and may even be high fidelity proofreading polymerases with 3'-5' exonuclease activity, but it should be noted that the enzymes required and used in Fahy's method exhibit high extension efficiencies in a primer extension reaction.

More specifically, although the Office Action notes that Fahy's method may use a Pfu polymerase, the reaction conditions in Fahy's method are significantly different from the reaction conditions of the present method, and Fahy's analysis of obtained results is significantly different from the analysis provided in the present invention. Although Fahy makes reference to Pfu DNA polymerase, the description of his method, and the support provided by his data refers to an exo-Pfu enzyme (page 32, line 32; page 35, line 15). Fahy's method does not provide support for the use of, for example, a high fidelity, low efficiency enzyme, such as exo+Pfu (i.e. Pfu) because Fahy's method teaches a high efficiency extension reaction.

In addition, by using the naturally specific and mispair extension using a specific enzyme, such as, Pfu with 3'-5' exonuclease activity, the products can be analyzed with automated DNA fragment length polymorphism analysis software in the OpenGen automated DNA sequencing system to identify the genotype-specific profile and to quantify the proportion of each genotype in a mixed genotypes infection. Accordingly, although Fahy contends that his method may be automated, the background or runthrough present in Fahy's method does not permit an automated or semi-automated process. Therefore, since the problems and deficiencies of Fahy's method are overcome by the present method, the PSMEA method has become an automated or semi-automated genotyping system (S-PSMEA), as claimed in claim 9. This additionally distinguishes the present invention from Fahy.

Therefore, unlike the teachings of Fahy, which compares primer extension length, the present method may determine a single point mutation or variation by a positive (+) or negative (-) reaction. The only approach Fahy provides for detection of a single point mutation is to use exo+ULTma and ddNTPs. This however differs from the teachings of the present invention since exo+ULTma is a high fidelity, high efficiency enzyme and the addition of ddNTPs are required in Fahy's reaction. Such conditions allow for misincorporation in the extension reaction, which, along with the presence of ddNTPs, will result in high background or

runthrough in the reaction, neither of which are considered in Fahy's analysis. This too is another difference between the present method and Fahy's method.

In connection with the objections to claims 3, 4, 5, 6, 7, 9 and 10, the applicant notes that claims 3, 4, 5, 6, 7, 9 and 10 depend from claim 1. Consequently, the applicant submits that in light of the above noted comments in connection with Fahy, and the amendments presently effected to claim 1, dependent claims 3, 4, 5, 6, 7, 9 and 10 are not anticipated by Fahy. Accordingly, reconsideration of the objections raised in the Office Action is respectfully requested.

The applicant therefore submits that, in view of the above noted comments, the subject matter of the present claims has not been previously disclosed, and is not anticipated with respect to the Fahy reference. The rejections of claims 1-10 are thereby transversed.

The applicant further submits that new claims 13, 14 and 15 are fully supported by the specification of the application as filed.

Rejection under 35 U.S.C. §102(a)

Claims 1-7, 9-11 have been rejected under 35 U.S.C. §102(a) as being anticipated by Hu et al (Nucl. Acids Res., vol. 26, November 1, 1998).

In reply to the objection, the applicant respectfully submits that, as noted on the Filing Certificate, priority of the present application is claimed from Canadian Application No. 2,245,039, filed August 13, 1998.

The applicant presumes the Office Action cites the Hu et al reference against the present application under 35 U.S.C. §102(a), due to an inaccuracy noted in the filed Power of Attorney documents. The applicant notes that, due to a clerical error, the filed executed Power of Attorney document incorrectly notes a priority date of August 9, 1999, for the Canadian application. This clerical error presumably arose from inadvertently confusing the filing date of the Canadian priority application with the filing date of the corresponding International application PCT/CA99/00733, which was filed August 9, 1999. The applicant apologizes for this inadvertent oversight, and for the resulting confusion, and notes that a replacement Power of Attorney document, identifying the correct priority date, will be filed in due course.

Accordingly, since the Hu et al document was published (November 18, 1998) after the priority date of the present application (August 13, 1998), the Hu et al publication does not precede, cannot anticipate, the present application. Accordingly, the applicant respectfully requests reconsideration and withdrawal of the Office Action's rejection of claims 1-7 and 9-11 in this respect.
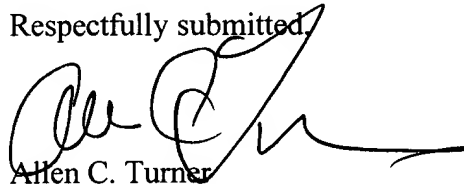
Claim Rejections - 35 U.S.C. §103

Rejection under 35 U.S.C. §103

Claims 11 and 12 have been rejected under 35 U.S.C. §103 as being unpatentable over Fahy in view of Resnick et al (U.S. Patent No. 5,527,669). In addition, claims 11 and 12 have been rejected under 35 U.S.C. §103 as being unpatentable over Fahy in view of Okamoto (U.S. Patent No. 5,550,016).

The applicant submits that in light of the above noted comments in connection with Fahy, and the presently submitted amended claims, dependent claims 11 and 12 would not have been obvious to one of ordinary skill in the art, at the time of the invention. Accordingly, it is respectfully requested that the Examiner's objections in this matter be withdrawn.

In view of the amendments to this application, and the comments noted hereabove, the application is now considered to be in a condition for immediate allowance. Favourable reconsideration and early issuance of a Notice of Allowance are requested.

Respectfully submitted,

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Date: March 18, 2002

ACT/bv

CLAIMS:

1. (Amended)

A primer-specific and mispair extension assay for determining genotype, said assay comprising:

   a)   extending a nucleic acid sequence from a patient sample with *pfu* DNA polymerase, using a primer specific for a genotype to be determined, and an incomplete set of dNTPs in the absence of ddNTPs, under suitable conditions for obtaining extension products of the primer, wherein at least one of the primer or the dNTPs is labeled;

   b)   characterizing the extension products; and

   c)   ~~comparing the extension products with known nucleic acid sequences of various genotypes for~~ analyzing the characterized extension products based on primer specific pairing and mispairing to ~~determining~~ determine the genotype of the nucleic acid sequence extended.

APPENDIX A

(CLEAN VERSION OF SUBSTITUTE SPECIFICATION
EXCLUDING CLAIMS AND ABSTRACT)

(Serial No. 09/782,361)

NOTICE OF EXPRESS MAILING

ou to enter
TS
5/20/02

Express Mail Mailing Label Number: _____

Date of Deposit with USPS: _____

Person making Deposit: _____

APPLICATION FOR LETTERS PATENT

for

PRIMER-SPECIFIC AND MISPAIR EXTENSION ASSAY FOR IDENTIFYING GENE VARIATION

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APPENDIX B

(MARKED-UP VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS AND ABSTRACT)

(Serial No. 09/782,361)

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: _____

Date of Deposit with USPS: _____

Person making Deposit: _____

APPLICATION FOR LETTERS PATENT

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PRIMER-SPECIFIC AND MISPAIR EXTENSION ASSAY FOR IDENTIFYING GENE VARIATION

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PRIMER-SPECIFIC AND MISPAIR EXTENSION ASSAY FOR IDENTIFYING GENE VARIATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   This application claims priority to, and is a continuation of, International Application No. PCT/CA99/00733, filed on 9 August 1999, designating the United States of America, the contents of which are incorporated by this reference, the PCT International Patent Application itself claiming priority from the Canadian Application Serial No. 2,245,039 filed 13 August 1998.

BACKGROUND OF THE INVENTION

[0002]   Field of the Invention: The invention relates to primer-specific and mispair extension assay for identifying gene variations, such as in different genotypes or subtypes of a given genotype.

[0003]   State of the Art: Current genotyping systems are technically complex, time-consuming and error-prone in the detection of a single nucleotide variation and low level heterozygotes.

[0004]   Despite current genotyping systems, such as restriction fragment length polymorphism analysis (RFLP), hybridization for example, line probe assay, ("LiPA"), selective DNA amplification by ~~PCR-type-specific~~PCR-type-specific primers (Okamoto, H., et al., *J. Gen. Virol.* **73**: 673, 678,_1992) and direct DNA sequencing, having been useful in general, some technical problems still remain and ~~limits~~limit their applications.

[0005]   Development of viral resistance to antiviral drugs used for treatment of HIV-1 infection is an important cause of treatment failure (Coffin JM., *Sciences*, **267**: 483-489, 1995). In addition, ~~drug-resistant~~drug-resistant mutations can give rise to selective cross-resistance to other antiviral drugs, which has limited the options available for alternative antiviral regimens (Cohen OJ, and Fauci AS., *N. Engl. J. Med.*, **339**: 341-243, 1998). Thus, genotypic drug resistance testing plays an important role in selecting an initial antiviral regimen and changing therapy with alternate regimens as the need arises. However, current genotyping assays, including direct DNA sequencing, yield information on only the predominant viral quasispecies due to the inability of these assays to detect low levels of viral variants (Günthard, ~~HF~~H.F., *et al.*, *AIDS Res. Hum. Retroviruses*, **14**: 869-

876, 1998). An ABI automated sequencer was able to detect mutants at levels of 10-50% in an artificially mixed sample only when the mixes were analyzed by editing the sequences manually. ~~Hybridization-based~~Hybridization-based high-density oligonucleotide arrays (GeneChip) (Deeks SG, Abrams DI., *Lancet*, **349**: 1489-1490, 1997) were less sensitive than the ABI and were able to detect mutations at levels of 25-75% in the mix (Günthard, ~~HF~~H.F., *et al.*, *AIDS Res. Hum. Retroviruses*, **14**: 869-876, 1998). Similar results were obtained using an automated DNA sequencer (Visible Genetics) (Hu YW, *et al.*, Reliable detection of mixed HCV genotype infections using a novel genotyping assay. 5th International Meeting on Viral Hepatitis C Virus and Related Viruses, Molecular Virology and Pathogenesis, Venezia, Italy, 1998). Line Probe Assay ("LiPA"), which uses reverse hybridization technology, is relatively rapid and could detect mutants at levels as low as 5% (Stuyver L, *et al.*, *Antimicrob. Agents Chemother.*, **41**: 284-291, 1997), but again, may not be suitable for detection of mixed genotypes because it may not give ~~no~~ results due to nearby polymorphisms that impair hybridization (Stuyver L, *et al.*, *Antimicrob. Agents Chemother.*, **41**: 284-291, 1997). Moreover, in 40% of the samples tested, LiPA failed to yield correct results for some of the ~~drug-resistance~~drug-resistant mutations (Puchhammer-Stockl E, *et al.*, *J. Med. Virol.*, **57**: 283-289, 1999). Population based sequencing, for example, cloning and sequencing, is the gold standard method for detection of minor ~~drug-resistant~~drug-resistant mutants. Unfortunately, it is impractical for clinics and large cohort studies.

[0006]   For most indirect DNA sequencing genotyping systems, a common weakness is that they are not as accurate as direct DNA sequencing analysis, particularly for detection of a single nucleotide mutation or variation, resulting in considerable instances of errors or inconsistent results (Andonov, A., *et al.*, *J. Clin. Microbiol.* **32**: 2031-2034, ~~1994)~~; ~~(~~Tuveri, R., *et al.*, *Journal of Medical Virology* **51**: 36-41, ~~(1997)~~; Okamoto, H., *et al.*, *J. Virol. Methods* **57**: 31 45 002-16, 1996). Although direct DNA sequencing is the most reliable method for genotyping, this is not practical for large cohort studies.

[0007]   For example, International Publication No. WO 91/13075 describes a method for detecting variable nucleotides based on primer extension and incorporation of detectable nucleoside triphosphate using T7 polymerase for extending the primer. However, T7 polymerase does not have the proofreading activity and the 3'-5' exonuclease activity of ~~Pfu resulting~~*pfu* results in false positive or false negative reactions. Moreover, the method uses ddNTPs for terminating extension.

[0008] Another major limitation of all current genotyping systems, including direct DNA sequencing, is that they ~~can not~~cannot reliably detect low levels of heterozygotes (Tuveri, R., *et al.*, *Journal of Medical Virology* **51**: 36-41, 1997); (Lau, J.Y., *et al.*, *J. Infect. Dis.* **171**: 281-289, 1995); (Forns, X., *et al.*, *J. Clin. Microbiology.* **34-10**: 2516-2521, 1996); or mixed genotype infections (Tuveri, R., *et al.*, *Journal of Medical Virology* **51**: 36-41, 1997); (Lau, J.Y., *et al.*, *J. Infect. Dis.* **171**: 281-289, 1995); (Forns, X., *et al.*, *J. Clin. Microbiology.* **34-10**: 2516-2521, 1996).

[0009] International Publication No. WO 96/30545 discloses a method for simultaneously analyzing a genetic mutation and a corresponding wild-type sequence within a sample. The method utilizes ddNTPs for terminating primer extension. However, the use of ddNTPs increases the background of the assay, reducing its sensitivity and capacity to detect low levels of heterozygotes, similarly as in WO 91/13075.

[0010] HCV was recognized as the major etiologic agent of blood borne non-A, non-B hepatitis soon after the virus was identified in 1989. As an RNA virus, HCV shows great genetic variability, resulting in the existence of types, subtypes and quasispecies. At present, 11 types and at least 50 subtypes have been described. However, types 1a, 1b, 2a, 2b and 3a have been found to be generally the most prevalent (Simmonds, P., *Hepatology* **21**: 570-582, 1995). Subtype 1b is the most common genotype found in Japan (Okamoto, H., *et al.*, *J. Gen. Virol.* **73**: 673, 678, 1992) and European countries while subtypes 1a and 1b are the most common genotypes in the United States (Lau, J.Y., *et al.*, *J. Infect. Dis.* **171**: 281-289, 1995) and Canada, (Andonov, A., *et al.*, *J. Clin. Microbiol.* **32**: 2031-2034, 1994). Viruses of various genotypes contain different antigenic properties, which have potentially important consequences for the development of a vaccine and for antibody screening tests. Also, the disease severity and response to interferon may be influenced by the virus types and subtypes (Simmonds, *Hepatology* **21**: 570-582, (1995). Subtype 1b was reported to be associated with a high severity of the disease and low response to interferon (Simmonds, *Hepatology* **21**: 570-582, (1995). It is apparent that a rapid, simple, accurate and inexpensive genotyping method is urgently needed.

[0011] Amplification refractory mutation system ("ARMS") (Newton, C.R., *et al.*, *Nucl. Acids Res.* **17**: 2503-2516), improved the methods used in the prior art for typing the five most common genotypes (Pistello, M., *et al.*, *J. Clin. Microbiol.* **32**: 232-234, 1994). ARMS was developed for PCR detection of any point mutation in DNA using *Taq* DNA polymerase (Newton, C.R., *et al.*, *Nucl. Acids Res.* **17**: 2503-2516) and is based on the principle that oligonucleotides

with a mismatched 3'-residue would not function as primers in PCR under controlled conditions. In some cases, however, the specificity of ARMS was ~~not sufficient enough~~insufficient to give a correct diagnosis. The problem with ~~non-specific~~nonspecific reactivities still remains with the type-specific primer PCR method for HCV genotyping, even with the improvement using ARMS.

[0012] It is apparent that the major cause of the nonspecific reactivities found in these assays is related to the use of *Taq* DNA polymerase due to its lack of 3'→5' exonuclease activity. This inaccuracy results in base substitutions, transitions, tranversions, frame shifts or deletion mutations during DNA synthesis. Consequently, mispairs can be frequently formed, and *Taq* polymerase would be able to continue synthesizing DNA by addition of the next correct nucleotide on the template (Lau, J.Y., *et al.*, *J. Infect. Dis.* **171**: 281-289, 1995). Even after reaching the end of the template, several more nucleotides can be added to the extended primer because most DNA polymerases, including *Taq* and retroviral reverse transcriptase (RT), have a ~~non-template dependent~~nontemplate-dependent DNA synthesis activity, for example, terminal deoxynucleotide transferase activity (Clark, J.M., *Nucleic Acids Res.* **16**: 9677; and Patel, P.H., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**: 549-553). Therefore, the nonspecific reaction cannot be avoided with either ARMS or the methods based on ARMS using *Taq* DNA polymerase.

[0013] Two thermostable DNA, polymerases *pfu* (P*yrococcus furiosus*) (Lundberg, K.S., *et al.*, *Pyrococcus furiousus. Gene* **108**: 1-6, 1991), and *TLI/Vent* (*Thermocococcus litoralis*), (Neuner, A., *et al.*, *Iarch. Microbiol.*, **153**: 205-207), exhibit 3'→5' proofreading exonuclease activity. This ensures a high degree of amplification fidelity during DNA polymerization. Unlike *Vent*, the *pfu* 3'→5' exonuclease activity peaks sharply at its optimal polymerization temperature (75°C to 80°C), minimizing undesirable primer-degradation activity (Lundberg, K.S., *et al.*, *Gene* **108**: 1-6, 1991). ~~pfu~~*Pfu* DNA polymerase also does not exhibit terminal deoxynucleotidyl-transferase (TDT) activity, which was reported to be involved in the high mutation rate of DNA during DNA polymerization.

[0014] It would be highly desirable to be provided with a simple assay or method to overcome many of these limitations of current DNA genotyping systems.

[0015] It would be highly desirable to be provided with genotypic assays that possess greater accuracy and sensitivity for detection of minor ~~drug-resistant~~drug-resistant subpopulations of HIV-1 in the early stages of resistance evolution.

DISCLOSURE OF THE INVENTION

[0016] One aim of the present invention is to provide a novel ~~primer specific~~primer-specific and mispair extension assay (PSMEA) for determining genotypes and subtypes.

[0017] Another aim of the present invention is to provide a ~~primer specific~~primer-specific and mispair extension assay (PSMEA) for detecting nucleotide variations in any known gene sequence using *pfu* DNA polymerase in the presence of an incomplete set of dNTPs, and only a single primer.

[0018] Another aim of the present invention is to provide a tool for reliable detection of mixed genotype infection.

[0019] Another aim of the present invention is to provide a sensitive tool for detecting low levels of ~~drug resistant~~drug-resistant mutants in patients being treated with antiviral drugs.

[0020] Another aim of the present invention is to provide a tool for accurate genotyping.

[0021] In accordance with the present invention, there is provided a primer-specific and mispair extension assay for determining genotype. The assay includes:

a) extending a nucleic acid sequence from a patient sample with a polymerase, more preferably a ~~PFU~~*pfu* DNA polymerase, using a ~~primer specific~~primer-specific for a genotype to be determined and an incomplete set of dNTPs, under suitable conditions for obtaining extension products of the primer, wherein at least one of the primer or the dNTPs is labeled;

b) characterizing the extension products; and

c) comparing the extension products with known nucleic acid sequences of various genotypes for determining the genotype of the nucleic acid sequence extended.

[0022] Preferably, characterizing the extension products includes separating the extension products by size. More preferably, characterizing the extension products further includes sequencing the extension products after separating the extension products by size.

[0023] Alternatively, the assay may further ~~includes~~include amplifying the nucleic acid sequence before extending the nucleic acid sequence from a patient sample.

[0024] Preferably, the incomplete set of dNTPs contains two or three different types of nucleotides.

[0025] In a preferred embodiment, the primer is labeled with a radioactive label or a fluorescent label.

[0026] In another embodiment, one of the dNTPs is labeled with a radioactive label or a fluorescent label.

[0027] In accordance with the present invention, there is provided a novel genotyping system, ~~primer specific~~primer-specific and mispair extension analysis (PSMEA) ~~using~~preferably using the unique 3' to 5' exonuclease proofreading properties of *pfu* DNA polymerase with an incomplete set of dNTPs and only a single primer.

[0028] Also in accordance with one embodiment of the present invention there is provided a primer-specific and mispair extension assay for determining genotype and detecting low level mixed genotype injections or heterozygotes. The assay includes:

a) extending a DNA sequence, amplified from a patient sample, with ~~PFU~~*pfu* DNA polymerase using a ~~primer specific~~primer-specific for a genotype to be determined and an incomplete set of dNTPs, under suitable conditions for extending the primer, wherein at least one of the primer or one of the dNTPs is labeled;

b) separating the extended DNA sequences obtained in step a);~~and~~

c) detecting the separated extended DNA sequences; and

d) comparing the extended DNA sequences with known DNA sequences of various genotypes for determining the genotype of the DNA sequences extended.

[0029] The primer may be end-labeled with a label or one of the dNTPs can be labeled with a label. The label can be a radioactive or fluorescent label.

[0030] Preferably, the steps a), b), c), and d) as described above are automated.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0031] FIG. 1 illustrates characteristics of ~~primer specific~~primer-specific and mispair extension by *pfu* and *Taq* DNA polymerase;

[0032] FIG. 2 illustrates nucleotide deletions and insertions by PSMEA with primer 6AR-1 and 6AR-2 extensions by *pfu* on templates 1a and 6a;

[0033] FIG. 3 illustrates a comparison of the sensitivity between PSMEA and direct DNA sequencing in detection of mixed genotypes;

[0034] FIG. 4 illustrates a typical profile of unlabeled primer extensions by *pfu* on HCV templates for types 1a, 1b, 2a, 2b, 3a and 3b using ^{32}P-labeled dCTP and dGTP;

[0035] FIGS. 5A and 5B illustrate the detection of HIV-1 ~~drug-resistant~~drug-resistant mutant V82A and wild type V82 using a universal primer and four sets of dNTPs;

[0036] FIGS. 6A and 6B illustrate the detection of HIV-1 ~~drug-resistant~~drug-resistant mutant L63P and wild type L63 using matched and mismatched pairs at the 3' end of the primers in PSMEA;

[0037] FIGS. 7A and 7B illustrate the detection of HIV-1 ~~drug-resistant~~drug-resistant L90M and wild type L90 using matched and mismatched pairs at the 3' end of the primers; and

[0038] FIG. 8 illustrates the sensitivity of PSMEA for detecting low levels of HIV-1 ~~drug resistant~~drug-resistant mutant with a defined mix of mutant V82I and wild type V82.

DETAILED DESCRIPTION OF THE INVENTION

[0039] In accordance with one embodiment of the invention, a novel ~~primer specific~~primer-specific and mispair extension assay (PSMEA) is provided for determining genotypes, and more preferably, Hepatitis C virus (HCV) genotypes and subtypes.

[0040] PSMEA is used to detect nucleotide variations in any known gene sequence using *pfu* DNA polymerase in the presence of an incomplete set of dNTPs. To test the feasibility of PSMEA, the 5' untranslated region ("UR") of the HCV genome, known for being highly conservative, was used as a model for analysis of the nucleotide variation in determining the type and subtype of the virus. The results demonstrate that PSMEA is a rapid, simple and accurate method for HCV genotyping.

[0041] In the 5' UR of the HCV genome, six major genotypes and some subtypes can be classified by the nucleotide variation in this region (Simmonds, P., *Hepatology* **21**: 570-582, 1995); (Stuyver, L., *et al.*, *J. Clin. Microbiol.* **34**: 2259-2266, 1996).

[0042] The PSMEA of the present invention is a simple method with great potential for accurately detecting nucleotide mutations and which may be used for detecting nucleotide variations in any known gene sequence.

[0043] PSMEA is based on the unique properties of 3'→5' proofreading activity in a reaction with an incomplete set of dNTPs. Under such reaction conditions in accordance with one embodiment of the invention, *pfu* is extremely discriminating in nucleotide incorporation and proofreading at the initiation step of DNA synthesis, allowing for an accurate detection of nucleotide variation and heterozygotes in PSMEA.

[0044]   It is known that under this reaction condition mispair formation and extension occur during DNA synthesis when using reverse transcriptase and DNA polymerases, including enzymes that exhibit 3'→5' proofreading exonuclease activity (Perinno, F., *et al.*, *Proc. Natl. Acad. Sci. USA.* **86**: 8343-8347, 1989); (Reha-Krantz, L.J., *et al.*, *Proc. Natl. Acad. Sci. USA.* **88**: 2417-2421, 1991). However, the frequency of mispair formation and extension depends on whether the polymerase possesses 3'→5' exonuclease activity, the concentration of nucleotide substrates and the composition of the mispairs (Reha-Krantz, *et al.*, *Proc. Natl. Acad. Sci. USA.* **88**: 2417-2421, 1991). Accordingly, the characteristics of primer-specificprimer-specific and mispair extension by thermostable DNA polymerases including *pfu* (*Pyrococcus furiosus*) (Lundberg, K.S., *et al.*, *Gene* **108**: 1-6, 1991), *Taq* (*Thermus aquaticus*), (Saiki, R.K., *et al.*, *Science.* **239**: 487, 1988), and *TLI/Vent* (*Thermocococcus litoralis*) (Neuner, A., *et al.*, *Iarch. Microbiol.*, **153**: 205-207) were further investigated. Several characteristics of primer-specificprimer-specific and mispair extension by *pfu* were observed and found to be useful for reliable detection of nucleotide variation, deletion and insertion, as well as heterozygotes.

[0045]   In accordance with a preferred embodiment of the invention, the accuracy was evaluated by assaying the nucleotide variations between genotypes in the 5' untranslated region (5' UR) of the hepatitis C virus (HCV) genome. Mixed infections with more than one genotype of HCV were used for comparison of the sensitivity of PSMEA with other assays in the detection of heterozygotes. The feasibility of the method of the present invention for large cohort studies was demonstrated by genotyping a total of two hundred and forty-fiveforty-five (245) HCV isolates. The results show that PSMEA is an extraordinarily accurate system for identifying nucleotide variation, genotyping and detecting heterozygous molecules, and is readily applicable for routine use.

[0046]   The design of PSMEA in accordance with a preferred embodiment of the invention is based on a single primer extension by the *pfu* DNA polymerase in the presence of only dCTP and dGTP, permitting accurate detection of nucleotide variations in the 5' untranslated region (5'UR) of the HCV genome. In accordance with one embodiment of the invention, the HCV genotypes from ninety-six (96) patients and blood donors with HCV infection were determined by PSMEA. Seventy-four (74) of the samples were also genotyped by either the line probe assay ("LiPA") or restriction fragment length polymorphism ("RFLP") methods. Genotypes were confirmed by nucleotide sequencing as required. HCV Isolates, including types and subtypes 1a,

1b, 2a, 2b, 2c, 3a, 3b, 4a, 5a, and 6a, were clearly identified by the PSMEA of the present invention. All of the types and subtypes determined by PSMEA were matched with those identified by LiPA or RFLP. Five (5) isolates of subtypes 1a and 1b that could not be typed by LiPA were clearly identified by the PSMEA of the present invention.

[0047]   The primers used in the present invention were designed to meet the following requirements: 1) the sequence of primer binding site on template should be a type- or ~~subtype specific~~subtype-specific; 2) primers used for PSMEA should exhibit a similar melting temperature. In accordance with the present invention, 11 primers were designed for HCV genotyping with PSMEA (Table 1).

[0048]   FIG. 1 illustrates preferred characteristics of ~~primer specific~~primer-specific and mispair extension by *pfu*.

[0049]   ~~In~~FIG. 1, column I illustrates ^{32}P-labeled primer extensions by ~~pfu~~*pfu* on template HCV genotype 1b in the presence of dCTP and dGTP (FIG. 1, columns I-1 and ~~1,~~I-2) used as markers for the length of primers and extended products. Column II illustrates the use of ^{32}P-labeled dNTPs instead of labeled primers for PSMEA under the same reaction conditions, showing the difference in primer extension between HCV genotypes 1a and 1b (FIG. 1, ~~column~~columns II-1, II-2, II-3 and ~~column~~II-4). Column III illustrates mispair formation and extension by *Taq* on template 1a with ^{32}P-labeled primers 1AB and 1BR (FIG. 1, ~~column~~columns III-1 and III-2). FIG. 1, parts I', II' and III' illustrate the nucleotide sequences extended on templates of HCV genotypes 1a and 1b. Primer 1AB extended on the antisense strand of templates 1a and 1b. X, XX and XXX represent the sites of nucleotide mismatches that terminated primer extension. → represents a nucleotide at the 3' end of the primer that is complementary to the opposite nucleotide in the template. (A), (C), (G) or (T) denotes the position of the nucleotide when a mispair is produced. - 113, -108 and -99 are the nucleotide positions in 5'UR of HCV. The underlined sequence indicates the primer binding sites. The signs in this figure are used for all other figures.

[0050]   A mismatched pair with nucleotide A at position -99 of the template HCV genotype 1a, that is opposite the first nucleotide to be incorporated at the 3' end of primer, could not be produced, and subsequently, aborted the primer extension by *pfu* in the presence of dCTP and dGTP (FIG. 1, column I-1). Under the same reaction conditions, the primer extended on the template of genotype 1b because nucleotide A becomes G at position -99 (FIG. 1, column I-2),

differentiating between genotypes 1a and 1b with this single nucleotide variation that is the only difference in the 5' UR between the two genotypes.

[0051]   FIG. 1 illustrates the alignment of the 5' UR nucleotide sequences of HCV subtypes 1a and 1b, showing the homologous sequence(----) and the difference of the nucleotide at position~~of~~ -99 (A for 1a and G for 1b). The primer extension stopped at position~~of~~ -99 where a mismatched pair(s) exists (X). The arrow (→) represents a nucleotide at 3' end of the primers that is complementary to the opposite nucleotide in the template. The parenthesis ( ) denotes that a nucleotide mispair occurred.

[0052]   The absence of mispair formation and extension at position -99 is due to *pfu* 3'→5' exonuclease proofreading activity that sharply peaks up at its optimal polymerization temperature, removing the mismatched nucleotide added at the initiation step of primer extension. It is apparently different from the 3'→5' exonuclease proofreading activity of other DNA polymerases such as pol-a and T4 in the primer mispair extension reaction. The mispair at the initiation site of DNA synthesizing can be produced with high frequency by these DNA polymerases in the primer mispair formation and extension reaction (Perinno, F., *et al.*, *Proc. Natl. Acad. Sci. USA.* **86**:8343-8347, 1989~~);~~ ~~(~~Reha-Krantz, L.J., *et al.*, *Proc. Natl. Acad. Sci. USA.* **88**:2417-2421, 1991).

[0053]   *Taq* DNA polymerase without 3'→5' exonuclease proofreading activities is used in virtually all ~~PCR-based~~PCR-based genotyping assays (Okamoto, H., *et al.*, *J. Gen. Virol.* **73:** 673, 678, ~~(~~1992~~);~~ ~~(~~Newton, C.R., ~~et al.~~*et al.*, *Nucl. Acids Res.,* **17:** 2503-2516). The unreliability caused by ~~cross-reactivity~~cross-reactivity and wrong priming has been a big concern (Lau, J.Y., *et al.*, *J. Infect. Dis.* **171:** 281-289, 1995~~);~~ and Forns, X., *et al.*, *J. Clin. Microbiology.* **34-10:** 2516-2521, 1996). When *Taq* is used instead of *pfu*, with the other conditions of the assay being the same, the primer 1BR extension took place on template 1a despite a mismatched pair existing at primer extension initiation site (-99) (FIG. 1, column III-2). This clearly indicates that the major cause of these nonspecific reactivities is due to the infidelity of *Taq* during the initiation of DNA synthesis (Perinno, F., *et al.*, *Proc. Natl. Acad. Sci. USA.* **86:** 8343-8347, 1989).

[0054]   In addition, since most DNA polymerases including *Taq* and reverse transcriptase of retroviruses exhibit a ~~non-template-dependent~~nontemplate-dependent DNA synthesis activity, for example, terminal deoxynucleotide transferase activity ("TDT"), several more nucleotides can be added to the 3' end of the primer, for example, single strand DNA (Clark, J.M., *Nucleic Acids Res.* **16:** 9677~~);~~ ~~(~~Patel, P.H., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91:** 549-553). Thus, *Taq* would be able

to continue synthesizing DNA by addition of a correct paired nucleotide next to the mispaired nucleotide, in particular, to the nucleotide in the A:C mispair that can be formed and extended more efficiently than other mispairs (Perinno, F., *et al.*, *Proc. Natl. Acad. Sci. USA.* **86**: 8343-8347, 1989);; (Newton, C.R., et al., *Nucl. Acids Res.* **17**: 2503-2516). A DNA strand with mismatched nucleotides would be generated and then act as a template that would be subsequently re-amplified, generating a large number of non-specificnonspecific DNA molecules. Therefore, the nonspecific reaction can notcannot be completely avoided with the methods based on DNA amplification with primer-specific PCR using *Taq* DNA polymerase. In contrast, in PSMEA, only a single primer is used, so that the extended primer cannot be re-amplified, and any mispairs, including A:C in front of the 3' end of the primer, could completely stop primer extension. *TLI/Vent* DNA polymerase exhibits 3'→5' proofreading exonuclease activity;; however, unlike *pfu*, *TLI/Vent* severely degraded single-strandedsingle-stranded DNA, for example, a primer, in PSMEA.

[0055]   In FIG. 1, column I-1, primer extension was performed using *pfu* with labeled primers (FIG. 1, column I-1), *pfu* with labeled dNTPs (FIG. 1, column II-1), and *Taq* with labeled primers (FIG. 1, column III-1). The symbol ↑ represents where the nucleotide incorporation starts. The symbol (X) denotes a stop of primer extension caused by the nucleotide mispair(s).

[0056]   The second feature of specific and mispair extension by *pfu* is that one correct pairing followed by more than one mispair in front of the 3' end of the primer is not sufficient for primer extension (FIG. 2, columncolumns I-1 and column I-2), at. At least two consecutive correct pairings are required (FIG. 2, columncolumns I-4 and column II-2). This provides a means to identify nucleotide deletion and insertion as well as multiple nucleotide variations or mutations in PSMEA.

[0057]   In FIG. 2, Primer 6AR-1 could not be extended with template HCV genotype 1a when using either dCTP plus dTTP, or dCTP plus dGTP as substrates due to a CA deletion (..), resulting either in a mispair at nucleotide position -145 in front of the 3' end of primer (FIG. 1, column I-1) or in only one matched pairing at the position (FIG. 1, column I-2). Primer 6AR-1 could not be extended on template HCV genotype 6a when using dCTP and dGTP (FIG. 1, column I-3). However, the primer was extended by three bases with template HCV genotype 6a using dGTP and dTTP which matched the nucleotides in the CA insertion (CA) in template HCV genotype 6a (FIG. 1, column I-4). Primer 6AR-2 was extended when using template HCV genotype 6a (FIG. 1, columncolumns II-1 and column II-2), but not with template HCV genotype

1a using either dATP, dCTP and dGTP or only dGTP (FIG. 1, ~~column~~columns II-3 and ~~column~~II-4). The symbol ↔ denotes the removal of the first nucleotide mismatched at the 3' end of the primer.

[0058] As seen in FIG. 2, there is a unique CA insertion in the 5' UR of HCV genotype 6a. Use of the CA insertion can differentiate between HCV genotype 6a and other genotypes. In the instant application, the CA insertion is used as an example to show how nucleotide deletion and insertion could be identified. Based on the flanking nucleotide sequence of the CA insertion, two primers were designed. A first primer 6AR-1, located in the region before a CA insertion in HCV genotype 6a, was extended only when using the correctly paired dNTPs, for example, dGTP and dTTP (FIG. 2, column I-4), but not those dNTPs (dCTP and dGTP) (FIG. 2, column I-3) that mismatched with the nucleotide A in the CA insertion. The second primer 6AR-2 was designed with the first nucleotide at its 3' end being matched with the first nucleotide A in the CA insertion of template HCV genotype 6a. Thus, the primer extended two bases or 11 bases on template HCV genotype 6a, depending on the substrates of dNTPs used (FIG. 2, columns II-1 and II-2), but not on template HCV genotype 1a due to a CA deletion that results in a 3' mispaired residue being removed after primer binding (FIG. 2, columns II-3 and II-4).

[0059] In accordance with the present invention, multiple nucleotide variations or mutations can be identified. For example, a unique TCA motif that is specific to HCV genotype 3a and 3b could be identified and is used for differentiation between the two genotypes and other genotypes.

[0060] Like mispair formation and extension by T4 DNA polymerase (Wilber, J. C., *et al.*, M. S. Reverse transcriptase-PCR for hepatitis C virus RNA, p. 327-331.~~In:~~ D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications~~:,~~ American Society for Microbiology, Washington D.C. 1993), any two or more consecutive mispairs could completely terminate primer extension by *pfu* because of its 3'→5' proofreading activity (FIG. 1, column II-2 and FIG. 2, columns I-3 and II-2). It was also found that two or more mispairs, separated by one or two correct base pairs (FIG. 1, column II-1 and FIG. 2, column II-1) could also terminate primer extension by *pfu*. Use of the termination point caused by these mispairs, as well as ~~primer specific~~primer-specific and mispair extensions on templates by *pfu*, provided reliable information on nucleotide sequence in the given region of the 5' UR of HCV.

[0061] The highest molecular weight band shown on the sequencing gels represents the longest sequence extended to the termination point that is specific to each of the HCV genotypes. As indicated in FIG. 1, column II, for example, the highest band represents the primer 1AB extended to the nucleotide C at nucleotide position -100 before the termination point at position -99 and -98 that would produce two mispairs with the two adjacent nucleotides A in template HCV genotype 1a, while the highest band represents the primer extended to the nucleotide G at -99 before the termination point at position -98 and -96 in template HCV genotype 1b, showing the single nucleotide difference between HCV genotypes 1a and 1b. This is another way to identify a single point mutation or variation in PSMEA in accordance with the present invention.

[0062] Whatever dNTPs, for example, one two or three of the four dNTPs, are chosen, they must follow the "instruction" with the characteristics of ~~primer-specific~~primer-specific and mispair extension by *pfu*.

[0063] The nucleotide incorporation rate of *pfu* is one fifth (1/5) of that of *Taq*. Thus, *pfu*-based PCR applications require a minimum extension time of 2.0 minutes/kb. The efficiency of nucleotide incorporation by *pfu* is high enough for PSMEA in which only less than 20 bases of extension are required per cycle in the reaction. Over 50% of the excess primer with less template (molecular ratio of primer and template 10:1) could be extended in a 20 cycle reaction, generating strong signals with either ^{32}P-labeled primer or dNTPs. Thus, PSMEA offers not only an advantage of superior accuracy over current indirect DNA genotyping systems, but an extraordinary sensitivity for detection of mixed infections with different genotypes of HCV.

[0064] Direct DNA sequencing is routinely used as the gold standard method for confirmation of the results from other assays (Tuveri, R., *et al.*, *Journal of Medical Virology* **51**: 36-41, 1997); (Forns, X., *et al.*, *J. Clin. Microbiology*. **34-10**: 2516-2521, 1996). In the present application, the sensitivity of PSMEA for identifying mixed infections was evaluated with direct DNA sequencing methods. In an experiment, the cDNAs (PCR products) from HCV genotypes 1b and 2a isolates were mixed in different proportions to mimic HCV mixed infections and heterozygotes. FIG. 3 shows that HCV genotype 2a could be clearly identified by direct DNA sequencing only when it reached to 50% in proportion in the mix. When HCV genotype 2a molecules consisted of less than 25%, only some of nucleotide variation, points could be manually recognized; but was not conclusive for the identification of which genotype it was. However, HCV

genotype 2a consisting as low as about 3% in the mix was clearly detected by PSMEA, showing approximately 10 times more sensitivity than the direct DNA sequencing system.

[0065] To further confirm the sensitivity of PSMEA, three samples identified having two or three genotypes by PSMEA were analyzed by direct DNA sequencing (FIG. 3, column B) and the reverse hybridization method. Results reveal that the presence of HCV genotype 2b was confirmed by the direct DNA sequencing method, but not by the reverse hybridization method in the sample containing 1a as dominant population.

[0066] FIG. 3 presents autoradiography results in column A and the date of a computer analysis of automated sequencing in column A'. FIG. 3, part A, illustrates the different proportions of genotypes 1b and 2a: 0% to 100% (I), 50% to 50% (II), 75% to 25% (III), 87.5% to 12.5% (IV), 93.75% to 6.25% (V), and 96.875% to 3.125% (IV) in the mix analyzed with PSMEA FIG. 13, part A and direct DNA sequencing FIG. 13, part A'. Two HCV genotypes, 1a and 2b, were identified in a thalassaemia patient sample by PSMEA FIG. 13, part B. N in FIG. 13, part B represents the negative reaction with primer 6AR and the sample from the thalassemia patient.

[0067] To evaluate the feasibility of PSMEA for large cohort studies, a total of two hundred and ~~forty-five~~forty-five (245) samples from HCV seropositive blood donors and patients with chronic hepatitis were genotyped by this assay. The genotypes determined by PSMEA included 1a, 1b, 2a, 2b, 2c, 3a, 3b, 4, 5a and 6a. The typeable rate of these samples with PSMEA was 95.5%. Eighty (80) of them were also typed with other indirect or direct DNA sequencing genotyping methods. The genotyping results from PSMEA were in 90-100% concordance with that from other genotyping methods including LiPA , RFLP and direct DNA sequencing. Less than 5% of untypeable samples were sequenced, indicating that those isolates being untypeable by PSMEA were either HCV mutants or unclassified genotypes. Results have proven a great utility of PSMEA for large cohort studies on viral genotyping.

[0068] PSMEA has been further developed with an automatic and colorimetric format, creating a great capacity to quickly genotype a large number of HCV isolates not previously possible. Therefore, PSMEA has a great potential for identifying nucleotide variations and heterozygotes in many areas such as virology, bacteriology, human genetics, epidemiology and legal medicine.

[0069] When a ~~type~~type- or subtype-specific sequence is available for designing the primer, for example primers 2A and 3A, no ~~cross-reativities~~cross-reactivities with other genotypes

are observed in PSMEA. Consistent results were obtained with primers 1AB, 2B, 2C, and 3R (Table 2).

[0070] Primer 1AB is universal for HCV genotypes 1a and 1b (FIG. 1, column III), differentiating the two genotypes from other genotypes except genotype 6a due to the homologous sequence in the region -131 and -99 between genotypes 1a/1b and 6a. Fortunately, a nucleotide A in the unique CA insertion between the nucleotide positions -145 and -144 in the 5' UR of 6a could be used as a first paired nucleotide at the 3' end of primer 6AR, thus, the primer specifically extended on template 6a, but not on other genotypes (FIG. 2, column I).

[0071] The primer could be bound on other genotypes, but could not be extended due to the CA deletion in these genotypes as compared with the insertion in 6a 5'UR. It was thus found that, for example, the primer 6AR-1 could not be extended because there was a single C at position -145 which could be paired in presence in the presence of dTTP and dGTP, followed by three Ts in the template HCA genotype 1a, in the reaction (FIG. 2, column I-1). However, the primer extended on template 6a in the same reaction conditions due to the presence of the two consecutive pairings C:G and A:T at positions between -145 and -144 (FIG. 2, column I-4). Accordingly, the primer 6AR2 could be extended longer on template 6a when using three dNTPs (dATP, dGTP and dCTP), showing a stronger signal to differentiate 6a from other genotypes (FIG. 2, column II-1). In the same manner, primer 5AR designed with a nucleotide variation at -236 was subtype specificsubtype-specific.

[0072] As shown in Table 2, primer 3B, that was originally designed for identification of genotype 3b, exhibited cross-reactivitycross-reactivity with 4e in PSMEA due to the homologous sequence in the region -175 to -149 chosen between the two genotypes. Thus, a small region (-99 to -79) that contains ana unique TCA motif in 5'UR of genotypes 3a and 3b was used for designing the primer 3R. Thus, by using primers 3A and 3R, genotypes 3a and 3b could be differentiated from other genotypes. Similarly, three genotypes 1a, 1b and 6a could clearly be identified with primer 1AB , 1BR and 6AR that exhibited a cross-reactivitycross-reactivity with 1AB. A total 11 genotypes, including six major genotypes (1a, 1b, 2a, 2b, 3a, 3b) and some ofuncommon genotypes (2c, 5a, 6a, 4a and 4e), in Canada could be identified by PSMEA using 11 primers (Table 2).

[0073] Extension of the mismatched 3' termini of DNA is a major determinant of the infidelity of the DNA polymerases that have no 3'→5' exonuclease activity, (Pistello, M. *et al.*, *J. Clin. Microbiol.* **32**: 232-234). With the PSMEA of the present invention, by using 3'→5'

exonuclease proofreading activity, high detection specificity for nucleotide mutation or variation in a known gene has been achieved. A significant advantage of this assay is that a single nucleotide variation, deletion and insertion can be accurately detected. In the genome of many natural virus mutants or ~~drug-resistant~~drug-restistant mutants, there may be only a single nucleotide mutation that has potential genetic or clinical significance. For example, a substitution C for A at nucleotide position 1814 that destroys the precore initiation codon, will prevent production of HBVeAg. In some ~~of drug-resistant~~drug-resistant mutants, a single nucleotide mutation could cause a failure of an antiviral therapy. PSMEA of the present invention can thus be used for rapid screening of those mutants.

[0074] A single point mutation could be associated with genetic disease in ~~human~~humans, such as a single point mutation, resulting in an amino acid substitution (C282Y) in the gene, *HLA-H* for haemochromatosis, which was reported to be involved in iron metabolism disorder. Such single point mutations are frequently found in viruses. For example, a substitution C for A results in loss of precore initiation codon of hepatitis B virus, preventing e-antigen (~~HBeAg~~HBVeAg) synthesis.

[0075] The PSMEA of the present invention can be modified so as to replace radioactivity by a detectable label. Such a ~~non-radioactive~~nonradioactive assay could be in the form of a colorimetric PSMEA in a microtiter-plate format.

[0076] The present invention will be more readily understood by referring to the following examples, which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I
DETERMINATION OF HCV GENOTYPES

[0077] Primer extension reactions contained 20 ng primer, 20-30 ng PCR product, 20 mM of each dCTP and dGTP, 10 mCi of each ^{32}p-labeled dCTP and dGTP, 1.25 units *pfu* DNA polymerase and 10 ml 10X *pfu* reaction buffer (PDI). When 5'-end ^{32}p-labeled primers were used, the ^{32}p-labeled dCTP and dGTP were omitted, and 100 μM of each dCTP and dGTP were used in the reactions. The primer extensions were performed in a reaction volume of 100 ml in a thermocycler (Perkin Elmer, GeneAmp 9600). Twenty (20) cycles consisting of a denaturation step at 94°C for 20 seconds, an annealing step at 64°C for 20 seconds and an extension step at 72°C for 35 seconds were performed. One microliter of the primer extension products was mixed with 1 μL

of the sequencing stop solution (Pharmacia Biotech) and electrophoresed on 8% polyacrylamide 8M urea TBE gels for one hour. Extension products were visualized by autoradiography.

[0078] For PCR amplification and sequence analysis, viral RNA was isolated from 100 ml of serum by treatment with RNAzol B (Biotecx Laboratories, Houston, Tex.) as previously described in Wilber, J. C., Johnson, P. J., and Urdea, M. S. Reverse transcriptase-PCR for hepatitis C virus RNA, p. 327-331. In, and in D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington D.C. 1993. RT-PCR was performed as described by Bernier *et al.* (Bernier, L., *et al.*, *J. Clin. Microbiol.* **34:** 2815-1818), with a set of primers that target highly conserved domains within the 5'-UR. The nucleotides and primers were removed from the PCR products with the QIAquick™ PCR Purification Kit (QIAGEN), using the procedure recommended by the product supplier. These purified PCR products were used for primer extension and automated sequencing analysis.

[0079] HCV isolates from patients with HCV infection were typed either by the improved Inno LiPA kit II™ using the procedure provided by the supplier (Innogenetics N.V., Belgium) or by RFLP analysis. For RFLP analysis, HCV genotypes were determined by cleavage of the PCR products with restriction enzymes BstNI, Bsr, Hinfl, Maelll, Haelll, BstUl and ScrFl. Digests were analyzed by gel electrophoresis and ethidium bromide staining.

EXAMPLE II

PRIMER DESIGN AND PSMEA PROCEDURE

[0080] In accordance with a preferred embodiment of the invention, the development of PSMEA is based on a single primer extension in the 5'-UR of the HCV genome using *pfu* DNA polymerase in the presence of only dCTP and dGTP in the reaction. Thus, under these reaction conditions, primer extension occurs when only the G and/or C nucleotides are added immediately downstream of the 3' end of the primer, since the incorporation of A or T nucleotide would in many instances be prevented by the 3'-5' exonuclease activity of *pfu*. The primers were designed to meet the following requirements:

1. They must have type-specific sequences; and

2. All primers used for PSMEA should exhibit a similar melting temperature.

The nucleotide sequences of the primers used in this study are shown in Table 1. In the PSMEA procedure, the primer extension reactions contained 20 ng primer, 20-30 ng PCR product, 20 mM of each dCTP and dGTP, 10 mCi of each ^{32}P-labeled dCTP and dGTP, 1.25 units *pfu* DNA polymerase and 10 ml 10X *pfu* reaction buffer (Stratagene). When 5'-end ^{32}P-labeled primers were used, the ^{32}P-labeled dCTP and dGTP were omitted, and 100 μM of each dCTP and dGTP were used in the reactions. The primer extensions were performed in a reaction volume of 100 ml in a thermocycler (Perkin Elmer, GeneAmp 9600). Twenty cycles of 94°C for 20 seconds, 64°C for 20 seconds and 72°C for 35 seconds were performed. One microliter of the primer extension products were mixed with 1 μL of the sequencing stop solution (Pharmacia Biotech) and electrophoresed on 8% polyacrylamide 8M urea TBE gels. Extension products were visualized by autoradiography.

EXAMPLE III

PCR AMPLIFICATION AND SEQUENCE ANALYSIS

[0081] Viral RNA was isolated from 100 ml of serum by treatment with RNAzol B (Biotecx Laboratories, Houston, Tex.) as previously described in Wilber, J. C., Johnson, P. J., and Urdea, M. S. Reverse transcriptase-PCR for hepatitis C virus RNA, p. 327-331. ~~In,~~ and in D. H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic Molecular Microbiology: Principles and Applications. American Society for Microbiology, Washington D.C. 1993.~ RT-PCR was performed as described by ~~Bernier et al.,~~ Bernier, L., *et al.*, *J. Clin. Microbiol.* **34**: 2815-1818, with a set of primers (see Table 1) that target highly conserved domains within the 5'-UR. If a second round PCR was necessary to provide sufficient cDNA for PSMEA, a pair of nested primers (sense primer -211 to -192 and antisense primer -91 to -74) was used. The nucleotides and primers were removed from the PCR products with the QIAquick™ PCR Purification Kit (QIAGEN), using the procedure recommended by the manufacturer. These purified PCR products were used for PSMEA and for automated sequencing analysis.

EXAMPLE IV

GENOTYPING OF HCV ISOLATES BY LiPA AND RFLP

[0082] .HCV isolates from patients with HCV infection were typed either by the improved Inno LiPA kit II™ using the procedure provided by the supplier (Innogenetics N.V., Belgium) or by RFLP analysis. For RFLP analysis, HCV genotypes were determined by cleavage of the PCR

products with restriction enzymes BstNI, Bsr, Hinfl, MaeIll, HaeIll, BstUl and ScrFl, (Andonov, A., *et al., J. Clin. Microbiol.* **32**: 2031-2034, 1994). Digests were analyzed by gel electrophoresis and ethidium bromide staining.

EXAMPLE V

The accuracy and reliability of PSMEA

[0083]   To evaluate the accuracy and reliability of PSMEA, 51 HCV isolates from HCV infected individuals in Montreal, Canada, typed by RFLP analysis in the 5' UR (Andonov, A., *et al., J. Clin. Microbiol.* **32**: 2031-2034, 1994) were analyzed with PSMEA in a double blind study. The subtypes 2a and 2c were grouped together when RFLP was used for genotyping of the 51 isolates, since some 2c variants share common ScrFI cleavage sites with 2a variants.

[0084]   A primer designed with sequence -133 to -113 from this isolate has shown no cross-reactivity~~cross-reactivity~~ with other types described in the present study (Table 2), suggesting that most isolates determined as 2a/2c by RFLP have a typical ~~2a-specific~~2a-specific sequence, and that the primer 2A can discriminate the majority of subtype 2a from subtype 2c. The results from the analysis of these 51 isolates indicate that 100% of types and subtypes for the isolates determined by PSMEA were matched with the types and subtypes identified with RFLP. In these isolates, there were 14-1a, 14-1b, 5-2a, 5-2b, 1-2c, 10-3a, 1-3b, and 1-6a. LiPA was reported to reliably type the most common genotypes, including some subtypes (Simmonds, P., Variability of Hepatitis C. Virus. *Hepatology* **21**: 570-582, 1995). Fifteen (15) isolates were typed by LiPA and evaluated with PSMEA in accordance with the present invention. They include 5-1a, 6-1b, 1-2a, 1-2b, 1-3a, 1-3b. The results from PSMEA showed a 100% agreement with that from LiPA.

[0085]   A group of five (5) HCV isolates that failed to be typed or subtyped by other methods were clearly identified as 1a or 1b by PSMEA. The results were confirmed by direct DNA sequencing using their PCR products from the 5' UR region (-211 to -71) (FIG. 4), suggesting that results from PSMEA is reliable.

[0086]   In the present application, the majority of the samples were typed with PSMEA using ~~non-labeled~~nonlabeled primers and [32]p-labeled dNTPs. Results obtained using [32]p-labeled dNTPs with unlabeled primers showed the typical patterns of the primer extensions as seen in the results from the reaction with [32]p-labeled primers and ~~non-labeled~~nonlabeled dNTPs.

[0087]   Accordingly the new genotyping assay, ~~primer specific~~primer-specific and mispair extension assay (PSMEA), of the present invention was used to genotype HCV and to detect mixed infections.   A total of one hundred and ~~forty-six~~forty-six (146) HCV isolates were typed and analyzed with PSMEA, showing that nine of 110 isolates (8.2%) from HCV positive blood donors and six of 36 isolates (16.7%) from thalassaemia patients were found to contain more than one genotype. The results were confirmed and compared with other current assays including direct DNA sequencing and line probe assay (LiPA). PSMEA of the present invention was found to be more reliable than other assays in detecting mixed infection.

EXAMPLE VI

Feasibility of PSMEA for large cohort studies

[0088]   Some genotypes including 1a, 1b, 2a, and 2b show a broad geographical distribution, and the infection frequency of subtypes 1a and 1b can be over 50% in blood donors and patients with chronic hepatitis in the United States (Lau, J.Y., *et al., J. Infect. Dis.* **171**: 281-289, 1995), Canada (Bernier, L., *et al., J. Clin. Microbiol.* **34**: 2815-1818), and (Andonov, A., *et al., J. Clin. Microbiol.* **32**: 2031-2034, 1994), and most European countries (Simmonds, P., *Hepatology* **21**: 570-582. 1995), and (Stuyver, L., Rosseau, *et al., J. Clin. Microbiol.* **34**: 2259-2266, 1996). Other genotypes, such as 3a and 3b are less common than 1a and 1b in those countries. However, 3a seems to be quite frequently found in Canada. Genotypes 4, 5a and 6a are only found in specific geographical regions in the Middle East, South Africa and Hong Kong, respectively, but were also infrequently found in some areas of Canada. It is apparent that for each region, a strategy for genotyping a large number of HCV isolates by PSMEA has to be designed, based on the genotype distribution and infection frequency with the population. For example, in Canada, over 60% of HCV isolates are genotypes 1a, 1b, which can be identified by primers 1AB, 1BR. However, since genotype 6a was frequently found in some areas of Canada and primer 1AB was ~~cross-reacted~~cross-reacted with 6a, thus all isolates diagnosed as 1a should be retested with primer 6AR for screening of genotype 6a in the first round of testing with PSMEA. The ~~non-typeable~~nontypeable isolates using the three primers should be retested with primers 2A and 2B. Thus, genotypes 2a and 2b (over 15% of total isolates) can be determined in the second round of testing. The remaining ~~non-typeable~~nontypeable isolates should be screened with primers 3A, 3B and 3R. Genotypes 3a and 3b (over 15% of total isolates) can be identified in the third round of testing. After the third round of

testing, the rest of the isolates (less than 10% of total isolates) that cannot be typed by these primers would include some subtypes of type 4, subtype 5a, subtype 2c or other genotypes. Table 3 identifies the infection frequencies of the major HCV genotypes identified by PSMEA, indicating that it is practical and feasible for genotyping a large number of isolates for epidemioloy and clinical studies.

[0089]   All current genotyping assays such as those for HCV, including direct DNA sequencing, are not suitable for detection of mixed infections because they are designed for detection of the population-dominant genotype. As indicated in Table 3, HCV mixed infection rate was higher than expected. A reliable detection of HCV mixed infections by PSMEA in different populations with HCV infection is thus reported.

EXAMPLE VII

DETERMINATION OF HIV-1 MUTANTS

[0090]   HIV positive sequential plasma samples were collected from patients on the combination therapy with RT and protease inhibitors for greater than three months.

[0091]   Viral RNA from samples was purified using the QIAamp™ Viral RNA kit. The Pharmacia Biotech First-Strand cDNA Synthesis kit was then used for cDNA synthesis. PCR was performed to amplify the region containing the entire protease and RT regions of HIV-1. The PCR products were purified using the QIA quick™ PCR Purification kit for genotypic analysis with PSMEA, direct DNA sequencing and cloning.

[0092]   PSMEA was performed with the procedure described above. This assay has been developed as a semi-automated system. Accordingly, a Cy5.5 dye-labeled primer (at the 5'-end of the primer) was used instead of a ³²P labeled ^{32}P-labeled primer. Primer extension reactions contained 20 ng of 5'-end 5.5 dye-labeled primer, 20-30 ng of PCR product, 20 μM of each dNTP, 1.25 units of *pfu* DNA polymerase and 10 μL of 10X *pfu* reaction buffer (PDI). Primer extensions were performed in a 100 μL reaction volume in a thermocycler (Perkin Elmer, GeneAmp 9600). Twenty cycles of 94°C for 20 seconds, 64°C for 20 seconds and 72°C for 35 seconds were performed. One microliter of the primer extension products was mixed with 1 μL of the sequencing stop solution (Pharmacia Biotech) and electrophoresed on 6% polyacrylamide 8M urea TBE mini gels for 10 minutes. Extension products were analyzed by automated DNA fragment length

polymorphism analysis software in the OpenGene™ automated DNA sequencing system (Visible Genetics).

[0093]   Both direct DNA sequencing and population based sequencing were performed with an automated DNA sequencer (Visible Genetics) and DNA sequencing kits (Visible Genetics). For the population based DNA sequencing, the PCR products were directly cloned into the TOPO™ TA vector (Invitrogen Corp.). The TTV sequence was amplified from positive colonies of *Escherichia coli* using M13 primers (Pharmacia Biotech). The re-amplified PCR products were sequenced using the same procedure as used for direct DNA sequencing.

[0094]   Based on the principle of PSMEA, primers can be designed to be either universal for both wild type and mutants or type-specific, for example, specific to either wild type or mutants. In addition, many options can be explored with different sets of dNTPs and locations of primer binding. To test the feasibility of PSMEA for HIV-1 genotyping, the three most common and important mutation sites of codons 82 (V82), 63 (L63) and 90 (L90) were detected by PSMEA and direct DNA sequencing. FIGS. 5A and 5B show an example to indicate the primer design and expected base extensions using different sets of dNTPs for detecting mutation of V82A with a universal primer. In FIGS. 5A and 5B, each combination of dNTPs results in a different extension profile, providing a fingerprint for wild type and mutant. In FIG. 5A, extension ~~profile~~profiles for wild type and mutant are illustrated. In FIG. 5B, the peak profiles represent the number of bases extended as detected by automated DNA sequencing using fragment length polymorphism analysis software, with the first peak from left representing unextended primer. When using specific sets of ~~dNTP's;~~dNTPs, ATC, ATG, ACG and TCG, the primer will extend 3, 1, 0 and 2 bases with wild type. In contrast, extensions of 0, 1, 5, and 2 bases will occur with mutant. These results clearly indicate that the predominant population in this sample was V82A mutant, showing a pattern of 0, 1, 5 and 2 bases extended. However, in the presence of ATC, about 12% of the primer extended, indicating a low proportion of the wild type virus still remained in this patient. Apparently only two sets of dNTPs (ATC and TCG) were needed to determine the levels of mutant and wild type in a mix. In some cases, V82 could mutate to V82I instead of V82A. A primer was designed for detection of V82I mutation using a set of dNTPs (A, T, and C).

[0095]   It is more difficult to detect ~~drug resistant~~drug-resistant mutations at codon 63 than other sites because in this case there are 6 combinations of codons for Leucine (L63) and 4 codons for Proline (P63). Since a wild type/mutant mix would almost certainly be caused by a single base

substitution, only four variations should be expected, with only CTC/CCC (L63) and CTT/CCT combinations seen so far, with the former combination being the most common. To overcome this problem, a new type of primer was designed for PSMEA to detect different ~~drug-resistant~~drug-resistant mutations of L63 codon as follows: the target mutation point was included as part of the 3' end of two primers. The 3' end of one primer (PRO63L(G)-R) was a perfect match to the wild type, but not mutant (FIGS. 6A(1) and 6A(2)), while the 3' end of the second primer (PRO63P(G)-R) was a perfect match to the nucleotide sequence of mutant, but not wild type (FIGS. 6A(3) and 6A(4)). In FIG. 6A, the matched primers should have 4 and 14 bases extension on both L63 and L63P ~~((1)~~ and (3))~~)~~, and the primers with mispairs on L63P and L63 were expected to have no extension (FIG. 6A, ~~part~~parts (2) and ~~FIG. 6A, part~~(4)). In FIG. 6B, the primer extended either 4 and 14 bases (second and last peaks from the left) FIG. 6A, parts (1) and (3), or virtually not at all (FIG. 6A, parts (2) and (4)).

[0096] If a mismatch occurs on the 3' end of the primer, the ~~Pfu~~pfu will remove the mismatched nucleotides. A mix of 3 dNTP's was added to the reaction in which there are no dNTPs to correct the mismatches on the 3' end of the primer. Thus, primer extension occurs under these circumstances only if the 3' end of the primer was a perfect match to the target mutation point. This results in very little, if any, ~~non-specific~~nonspecific extension, even at annealing temperatures as low as 55°C. When dNTPs (A, T and C) were added, the matched primer was extended with 5 and 14 bases extensions (FIG. 6A, parts (1), (2), and (3) and FIG. 6B, parts (1), and (3)). However the mismatched primer could not be extended because the mismatched nucleotides at 3' end of the primer were removed with no dGTP to repair the mismatch (FIG. 6A, ~~part~~parts (2), ~~FIG. 6A, part~~and (4) and FIG. 6B, parts (2), and (4)).

[0097] Unlike protease codon 63 which has multiple combinations for L63 mutating to L63P, and codon 82 with V82 mutating to V82I/A/others, codon 90 has only one possible combination for L90 mutating to L90M (AAG to ATG). As a result, only one pair of primers is required for detection of this mutation point. FIGS. 7A and 7B show that the primer (PRO90L-R) was designed in a reverse direction for detection of L90. FIG. 7A illustrates the expected number of bases extended with matched and mismatched primers on wild type and mutant, respectively (1-4). In FIG. 7B, primers PRO90L-R and PRO90M-F on wild type were extended with 4 on wild type (1) and 8 bases on mutant (3).

[0098] As expected, the primer was extended with four bases (FIG. 7A, part (1) and FIG. 7B, part (1)). Under the same reaction conditions, the primer was not extended on mutant L90M due to the T:T mismatch at the 3' end of the primer (FIG. 7A, part (2) and FIG. 7B, part (2)). Another primer (PRO90M-F) was designed to detect low levels of ~~drug resistant~~drug-resistant mutant L90M. The primer was extended on L90M mutant with eight bases (FIG. 7A, part (3) and FIG. 7B, part (3)), but not on wild type due to the second to last nucleotide mismatch (A:A) at the 3' end of the primer (FIG. 7A, part (4) and FIG. 7B, part (4)).

[0099] In order to assess the sensitivity of PSMEA for detecting low levels of mutants in a viral population, an artificial mixing experiment was carried out. PCR products from the protease region of V82 wild type and the V82I mutant were mixed in the following proportions between mutant and wild type: 3.1% (1/32), 1.6% (1/64), 0.8% (1/128) and 0.0% (~~i.e.~~for example, 100% wild type). FIG. 8 shows that 0.8% mutant in the mix was clearly detected with PSMEA. The levels at 0.8%-3.1% were not detected by direct DNA sequencing. It has previously been reported that the mixed genotypes or quasispecies could be detected only when mixed genotype(s) reached levels over 25% (Deeks SG, Abrams DI., *Lancet*, 349: 1489-1490, 1997).

[0100] To confirm the sensitivity of PSMEA, a total of 32 isolates from patients being treated with antiviral drugs have been analyzed with both direct DNA sequencing and PSMEA for the presence of ~~drug resistant~~drug-resistant mutations in codon V82, L63 and L90 from the protease region of HIV-1. Table 4 shows a 100% concordance of the genotypic testing results between PSMEA and direct DNA sequencing for detection of predominant population of variants including mutants and wild type.

[0101] ~~Twenty-five~~Twenty-five (25) (78.1%) of 32 samples determined as being fully wild type (V82) were found to contain either mutants V82I or V82A at various levels (1-19%). Different levels (3-27%) of L63P mutant were detected by PSMEA in 100% (5/5) of the samples that were determined as purely wild type (L63) by direct DNA sequencing. However, in detecting L90M mutation, the results showed 86.7 % (13/15) concordance between the two methods.

[0102] One of the samples (AR283) was analysed using population based sequencing, direct DNA sequencing and PSMEA to further evaluate the accuracy of PSMEA in detecting low levels of ~~drug resistant~~drug-resistant mutants. Table 5 shows that PSMEA demonstrated greater concordance with population based sequencing, than direct DNA sequencing for monitoring minor variants. Seventy-six percent (76%) and twenty percent 20% of isolate from AR283 was found to be

V82I mutant and wild type V82, respectively, by PSMEA, but 100% mutant V82I by direct DNA sequencing. Population based sequencing showed that wild type truly existed at a level of 12.5% (4/32). For detection of predominant populations variants (mutant V82I and wild type L90) in the isolate, both direct DNA sequencing and PSMEA were 100% concordant with population based sequencing.

[0103] From the above, it is thus apparent that PSMEA is more sensitive than direct DNA sequencing for detection of low levels of ~~drug resistant~~drug-resistant mutants. The high degree of sensitivity of PSMEA should allow one to more precisely understand how early drug resistant mutations can develop, especially during antiretroviral therapy. For clinics, this may further aid in guiding clinicians in modulating therapeutic regimens before the appearance of phenotypic drug resistance or deterioration in clinical status. With early warning of drug specific mutations, agents can be chosen to impede resistance and minimize ~~cross resistance~~cross-resistance, yet still have an antiviral effect on the patient's dominant quasispecies. This, in turn, will hopefully delay therapeutic failure and increase survival.

SEQUENCE LISTING

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Table 1. Nucleotide sequence of the primers for PSMEA

Primers	Position	Sequence from 5 to 3
For PCR (universal)		
1st round	-302 to -278	CTC CCC TGT GAG GAA CTA CTG TCTT (sense) (SEQ. ID. NO. 1)
	-50 to -31	CTC GCA AGC ACC CTA TCA GG (Antisense) (SEQ. ID. NO. 2)
2nd round	-204 to -175	CCA TAG TGG TCT GCG GAA CCG GTG AGT ACAC (Sense) (SEQ. ID. NO. 3)
	-91 to -74	CCC AAC ACT ACT CGG CTA (Antisense) (SEQ. ID. NO. 4)
For PSMEA		
1AB	-131 to -111	CTC AAT GCC TGG AGA TTT GGG (SEQ. ID. NO. 5)
1	-76 to -157	CAC CGG AAT TGC CAG GAC GA (SEQ. ID. NO. 6)
1BR†	-98 to -78	ACA CTA CTC GGC TAG CAG TCT (SEQ. ID. NO. 7)
2A	-134 to -114	CCA CTC TAT GCC CGG TCA TTT (SEQ. ID. NO. 8)
2B	-128 to -108	TAT GTC CGG TCA TTT GGG CAC (SEQ. ID. NO. 9)
2C	-133 to -113	CAC TCT GTG CCC GGC CAT TTG (SEQ. ID. NO. 10)
3A	-175 to -157	ACC GGA ATC GCT GGG GTG A (SEQ. ID. NO. 11)
3B	-175 to -157	ACC GGA ATC GCC GGG ATG A (SEQ. ID. NO. 12)
3R†	-99 to -79	CAC TAC TCG GCT AGT GAT CTC (SEQ. ID. NO. 13)
5AR†	-236 TO -218	GGG GGT CCT GGA GGC TGT T (SEQ. ID. NO. 14)
6AR†	-145 TO -125	CAT TGA GCG GGT TTG ATC CAA T (SEQ. ID. NO. 15)

† Antisense primer

Table 2. Identification of HCV genotypes with each of the primers by PSMEA

Primers	HCV Genotypes										
	1a	1b	2a	2b	2c	3a	3b	4a	4e	5a	6a
Reactivities of the Primers											
1AB	+	+									+
1	+	+						+			
1BR		+						+	+	+	
2A			+								
2B			*	+							
2C					+						
3A						+					
3B							+	+	+	+	
3R						+	+				
5AR										+	
6AR											+
Criteria for determination of the genotypes by the positivities of the primers	1AB+	1BR+	2A+	2B+	2C+	3A+	3B+	1+	1BR+	5AR+	6AR+
	6AR	1AB+	2A+			3R+	1BR+	3B+			

*weak reaction

Table 3

The prevalence of HCV mixed genotype infections in different populations determined by PSMEA and direct DNA sequencing

	PSMEA	Direct DNA sequencing
Blood donors	7/72 (9.7%)	ND
Patients with chronic hepatitis C	15/80 (18.8%)	8/80 (10.0%)[#]
Thalassemia patients	6/36 (16.7%)	4/36 (11.1%)

[#] The samples determined to contain more than one genotype by direct DNA sequencing were 100% concordant with that determined by PSMEA. Since ~~direct~~ the direct DNA sequencing method is not sensitive enough to detect low levels (<25%) of mixed genotype infections, the percentage of mixed genotype infections determined by direct DNA sequencing is lower than what was found by PSMEA.

Table 4
Comparison of the sensitivity between direct DNA sequencing and PSMEA for detecting low levels
of drug resistant mutations

Sample ID	Direct DNA Sequencing			PSMEA						
	L63/P/C/L63T (W/M)	V82/I/A (W/M)	L90/M (W/M)	L63 (%)	L63P/L63C (%)	V82/V82I/V82A (%)			L90/L90M (%)	
4B	L63	V82	L90			91	7	2		
6B	L63P	V82	L90	3	97P	96	2	2		
7B	L63C	V82	L90			83	14	3		
8B	L63P	V82	L90	2	98P	89	3	8		
GL	L63P	V82	L90M			97	<1	2	7	93
JB	L63P	V82A/V	L90M/L			31	15	54	56	44
MN	L63P	V82A	L90M	0	100P	22	10	68	5	95
CA	L63P	V82	L90	0	100P	89	9	2	100	0
DS1	L63P	V82	L90			86	12	2		
DS2	L63P	V82	L90	3	97P	95	3	2		
GP	L63P	V82	L90			91	3	6	100	0
AS		V82A				14	4	82		
FP	L63P	V82	L90			90	7	3	100	0
M15-8	L63C	V82	L90	49	51C	93	5	2		
M15-10	L63	V82	L90	73	27C	90	6	4		
M17-17	L63C	V82V/I	L90	50	50C	71	27	2		
M17-22	L63C	V82	L90	48	52C	90	8	10		
M17-25	L63	V82	L90	88	12C	54	17	29		
M17-33	L63T	V82	L90			84	14	2		
M17-35	L63T	V82	L90			81	16	3		
M17-37	L63	V82	L90	94	6C	79	19	2		
M17-41	L63	V82	L90	97	3C	93	4	3		
M17-44	L63P	V82	L90M			93	5	2		
98027	L63	V82	L90	96	4P	89	9	2	99	1
V894713	L63	V82	L90			89	9	2	100	0
AR283	L63P	V82I	L90			20	76	4	100	0
AR929	L63P	V82I/V	L90			33	65	2	100	0
AR2429	L63P	V82V/I	L90			39	60	1	100	0
AP6423	L63L/P	V82	L90			90	8	2	100	0
AP6617	L63P	V82	L90			89	10	1	100	0
AP7010	L63P	V82	L90			90	9	1	100	0
AP7366	L63P	V82	L90			90	8	2	100	0

The percentage of mutant in a mix is calculated as follows:
% mutant = (% wild type extension + % mutant extension) x 100

Twenty-five (25) (78.1%) of 32 samples determined as being fully wild type (V82) were found to contain either mutants V82I or V82A at various levels (1-19%). Different levels (3-27%) of L63P mutant were detected by PSMEA in 100% (5/5) of the samples that were determined as purely wild type (L63) by direct DNA sequencing. However, in detecting L90M mutation, the

Table 5

Evaluation of the accuracy of PSMEA and direct sequencing for detection of low levels of variants with population based sequencing

Method	Genotypes			
	%V821	%V82	%L90M	%L90
Population based sequencing	87.5(28/32)*	12.5 (4/32)	0.0 (0/32)	100.0 (32/32)
Direct DNA sequencing	100.0	0.0	0.0	100.0
PSMEA	76.0	20.0	0.0	100.0

* Denotes that a total number of mutant or wild type found in 32 cloned PCR products sequenced.